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(54) Title: RAPID DETECTION OF BIOPOLYMERS IN STAINED SPECIMENS (57) Abstract <p>The present invention provides methods to detect the presence of a biopolymer in a previously stained specimen using novel <i>in situ</i> hybridization techniques. The method comprising assaying for the presence of cellular biopolymers, specifically kNA and DNA. The novel <i>in situ</i> hybridization procedure may be accomplished either in one or two (fixation followed by hybridization) steps. The methods of the present invention may be used to detect the presence of microorganisms, e.g., bacteria, viruses, fungi or cellular genes, e.g. oncogenes, tumor suppressor factors, or growth stimulatory factors in either previously stained cells or tissue.</p>		

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**RAPID DETECTION OF BIOPOLYMERS IN
STAINED SPECIMENS**

BACKGROUND OF THE INVENTION

5 1. Field of the invention.

The present invention relates to the field of *in situ* hybridization. More specifically, the present invention relates to the use of *in situ* hybridization assays as a means to detect as few as 1 copy of cellular biopolymer in a stained cell.

10 2. Description of the related art.

10 *In situ* hybridization provides a technique for the determination and quantitation of biopolymers such as nucleic acids (DNA and RNA) and proteins in tissues at the single cell level. Such hybridization techniques can detect the presence or absence of specific genes in tissues at the single cell level. *In situ* hybridization procedures may also be utilized to detect the expression of gene products at the single
15 cell level.

By the use of specific nucleic acid (RNA or DNA) probes, genetic markers for infection and other disease states may be detected. Certain genetic diseases are characterized by the presence of genes which are not present in normal tissue. Other diseased conditions are characterized by the expression of RNAs or
20 RNA translation products (i.e. peptides or proteins) which are not expressed in normal cells. Some disease states are characterized by the absence of certain genes or gene portions, or the absence or alteration of expression of gene products or proteins.

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Current methods allow the detection of these markers but are relatively time consuming and of limited sensitivity. Hybridization techniques are based on the ability of single stranded DNA or RNA to pair, i.e., hybridize, with a complementary nucleic acid strand. This hybridization reaction allows the development of specific probes that can identify the presence of specific genes (DNA), or polynucleotide sequences or the transcription and expression of those genes (mRNA).

Solution hybridization methods which require the destruction of the cell and the isolation of the nucleic acids from the cell prior to carrying out the hybridization reaction sacrifice the cellular integrity, spatial resolution and sensitivity of detection. *In situ* hybridization allows the detection of RNA or DNA sequences within individual cells. *In situ* hybridization yields greater sensitivity than solution hybridization by means of eliminating the dilution of a particular target gene, nucleic acid, or protein by the surrounding and extraneous RNA and DNA of other cells. *In situ* hybridization also allows for the simultaneous detection of multiple substances, i.e. genes, nucleic acids or proteins within individual cells, permitting the identification of a particular cell expressing a cellular gene or viral sequence. In addition, since *in situ* hybridization analysis is performed and quantitated for single cells, minimal sample and reagent is required.

Prior to the present invention, *in situ* hybridization procedures were only capable of detecting nucleic acids present at greater than ten copies per cell. Such procedures required at least 8 hrs. to over 14 days to perform. Prior *in situ* procedures were neither quantitative nor capable of performing multiple simultaneous detections. More recently, *in situ* hybridization procedures, disclosed in copending United States patent applications Serial No. 784,690, filed October 28, 1991, and Serial No. 668,751, filed March 13, 1991, have reduced the time to under four hours and increased the sensitivity to a single copy per cell.

Traditional laboratory methods for identifying bacteria have relied heavily on the use of either simple or differential microbiological tests such as the Gram stain, the growth on common (or exotic) diverse substrates, and, subsequently, the specific use of biochemical tests. In recent years a strong trend in microbial systematics has emerged for the use of direct molecular characterizations, e.g., DNA

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base composition, DNA/DNA hybridization, gene sequencing, etc., to define taxa. These methods have revolutionized studies in bacterial evolution, but did little to identify a particular organism in a given environment or specimen. Thus, the processes of isolating pure cultures, and conducting the traditional procedures for culture identification, has continued largely unabated.

Recent efforts have attempted to develop methodologies for rapid identification of microorganisms. One of these has been the use of DNA probes specific for an organisms' 16S rRNA. However, the prior art is deficient in the lack of methods sensitive enough to detect 16S DNA, i.e., detect as few as seven copies of 16S DNA. S. Jinks-Robertson et al., In Cellular and Molecular Biology, 2:1367-1385 (1987). One advantage of using the 16S DNA is the huge data base of sequence information currently available. Almost 1000 16S DNA sequences have been documented for bacterial phylogenetic studies and this database includes a large variety of bacteria, including pathogens. From a technical perspective the 16S DNA sequence segments can serve as molecular probes, which will allow for species identification of any bacterium, whether viable, actively growing or non-viable, dormant organisms.

A number of different stains are used in cytologic and histologic specimens, for example, the papanicolaou stain is a common stain used on cells obtained from cervical specimens. Generally, only one slide is prepared from a patient and this slide is stained. If, for example, there are cells present on the stained slide which one suspects for HPV infection, the prior art does not provide a method for removing the coverslip and analyzing those suspicious cells by using *in situ* hybridization. Accordingly, an additional specimen must be obtained presenting the potential for missing the lesion in a subsequent sampling.

The prior art remains deficient in its inability to teach the use of *in situ* hybridization procedures in the detection of bacterial or viral DNA or mRNA or cellular genes in stained specimens. The application of *in situ* hybridization techniques to detect DNA or forms of RNA and mRNA and thus identify bacterial or viral strains and cellular genes present in stained histological or cytological samples is a long felt need and desire in this art.

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SUMMARY OF THE INVENTION

In one embodiment of the present invention, there is provided a method of detecting the presence of a biopolymer in a previously stained specimen having substantially intact cellular membranes. This "two-step" method comprises assaying for the presence of cellular biopolymers. Initially, the specimen may be heat fixed or fixed with a medium comprising a precipitating agent and/or a cross-linking agent. Subsequently, the fixed cells are contacted with a hybridization solution. The hybridization solution consists of a denaturing agent, hybridized stabilizing agent, buffering agent, a selective membrane performing agent and at least probe having a nucleotide sequence at least substantially complementary to a specific target nucleotide to be detected. The sample of cells is then incubated with the hybridization solution in the presence of at least one detectable label. This method is capable of detecting as few as a single biopolymer per cell.

In another embodiment of the present invention, there is provided a "one-step" method for the detection of the presence of biopolymers in a previously stained specimen having substantially intact membranes. The method comprising assaying for the presence of cellular biopolymers. Initially, the specimen is contacted with a medium comprising a denaturing agent, hybridized stabilizing agent, buffering agent, a membrane pore forming agent and at least one probe. Optimally, the medium may contain a fixative agent. The specimen is contacted under hybridizing conditions. Subsequently, the sample of cells is incubated with the medium in the presence of at least one detectable label. Duplex or triplex formation is detected by detecting the label without performing a pre-hybridization step for blocking non-specific binding of the probe and facilitating probe entry. This method is capable of detecting a single copy of a target biopolymer per cell.

In yet another embodiment of the present invention, there is provided a kit for determining the presence of a biopolymer in a previously stained specimen having substantially intact cellular membranes. The kit may be used for assaying cellular biopolymers. The kit comprises a hybridization solution comprising a denaturing agent, a hybrid stabilizing agent, a buffering agent, a membrane pore forming agent and optimally, a fixative agent. Optionally, the kit may comprise a

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supply of a probe selected so that the probe will hybridize with the suspect biopolymer to form a hybridized complex. Additionally, the kit may comprise a means for contacting said suspect specimen with the probe to form a hybridized complex and a means for measuring the presence of the labeled probe.

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BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 demonstrates the detection of HPV in a cervical cell previously stained with a papanicolaou stain.

Figure 2 demonstrates the simultaneous detection and differentiation of HPV types 16 and 18, in cells previously stained with a papanicolaou stain.

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Figure 3 demonstrates the detection of HPV in cervical tissue previously stained with hematoxylin and eosin.

Figure 4 demonstrates the detection of chromosomal DNA within cells previously stained by the Diff-Quick method.

DETAILED DESCRIPTION OF THE INVENTION

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The novel methods of the present invention may be used to detect a wide variety of microorganisms that are the cause of various pathophysiological states. For example, the methods of the present invention may be used to detect microorganisms that are the cause of pathophysiological states such as bacteremias, sexually transmitted diseases, diarrhea and respiratory diseases.

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The novel methods of the present invention may be used to detect a wide variety of bacteria, viruses and fungi. Representative examples of bacteria detectable by the methods of the present invention include *Streptococcus*, *Staphylococcus*, *Clostridium*, *Bacillus*, *Pseudomonas*, *Salmonella*, *Klebsiella*, *Bacteroides*, *Escherichia coli*, *Neisseria gonorrhea*, and *Chlamydia*. Representative examples of fungi include *Candida*, *Cryptococcus neoformans*, *Blastomyces dermatitides*, *Histoplasma capsulatum*, *Coccidioides immitis* and *Paracoccidioides brasiliensis*. Representative examples of detectable viruses include human papilloma, herpes simplex virus II, hepatitis, human immunodeficiency virus, influenza virus, parainfluenza virus and rota virus.

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Representative examples of spirochetes include *Treponema pallidum*, *Borrelia burgdorferi* and *Leptospira*. Representative examples of protozoa include

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Entamoeba histolytic, *Balantidium coli*, *Giardia lamblia*, *Leishmania tropica*, species of *Plasmodium*, *Trichomonas vaginalis* and species of *Trypanosoma*.

In contrast to the detection of a microorganism, the present methods may also be used to detect and quantitate the presence of cellular genes from previously stained mammalian specimens. Representative examples of such cellular genes include oncogenes, tumor suppressor genes and growth stimulating factors. Preferably, such oncogenes are neu (c-erb-B-2), c-ras, c-myc, and c-myb. Similarly, the present methods may be used to detect and quantitate tumor suppressor genes and stimulating growth factors. Examples of tumor suppressor genes include p53 and retinoblastoma. Representative examples of growth factors include colony stimulating factor-granulocyte/macrophage (CSF-GM), transforming growth factor (TGF- α), and epidermal growth factor (EGF). A person having ordinary skill in the art would comprehend that other cellular biopolymers may be detected and quantitated from a previously stained specimen by the methods of the present invention.

It is specifically contemplated that a wide variety of cell samples and tissue specimens may be employed in the techniques described in the present invention. The cell specimens include the following representative examples: cervical cells, bone marrow cells, hepatocytes, cerebrospinal fluid cells, blood cells, oral mucosa cells, lung cells and skin cells. Representative examples of tissue specimens include lymph node tissue, mammary tissue, cervical tissue, colon tissue, prostate tissue, cardiac tissue and brain tissue. In addition, fluids and exudates, e.g., urine, stool, sputum may be detected.

The cells and tissue specimens used in the method of the present invention may have been stained by any of the commonly used stains in cytology or histology. Examples of representative stains include a papanicolaou stain, a Wright stain, a Hematoxylin and Eosin stain and Diff-Quick.

Mounting Cells/Tissues

The first step in the *in situ* hybridization procedures could be the deposition of specimens onto a solid support. Specimens constitute any material which is composed of or contains cells or portions of cells. The cells may be living or dead, so long as the target biopolymer, i.e., DNA or mRNA, is largely unaltered

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and undamaged to the extent that it is capable of detection. The specimen should contain cells with substantially intact membranes. Although it is not necessary that all membranes of the cellular structure be intact, the membranes must be sufficiently preserved to allow: retention of the target biopolymer and introduction of the detecting probe to the site of the target biopolymer.

Techniques for depositing the specimens on the solid support will depend upon the cell or tissue type, and they may include, for example, standard sectioning of tissue or smearing or cytocentrifugation of single cell suspensions.

Many types of solid supports may be utilized to practice the invention. Supports which may be utilized include, but are not limited to, glass, Scotch tape (3M), nylon, Gene Screen Plus (New England Nuclear) and nitrocellulose. Most preferably glass microscope slides are used. The use of these supports and the procedures for depositing specimens thereon will be obvious to those of skill in the art. The choice of support material will depend upon the procedure for visualization of cells and the quantitation procedure used. Some filter materials are not uniformly thick and, thus, shrinking and swelling during *in situ* hybridization procedures is not uniform. In addition, some supports which autofluoresce will interfere with the determination of low level fluorescence. Glass microscope slides are most preferable as a solid support since they have high signal-to-noise ratios and can be treated to better retain tissue.

Cell Preparation for One-Step Method

In the one-step *in situ* hybridization procedure of the present invention, tissue samples are broken apart by physical, chemical or enzymatic means into single cell suspension.

A single solution is added to the cells/tissues (hereafter referred to as the specimen). This solution contains the following: optionally, a mild fixative, a chaotrope or other denaturing agent, a synthetic oligonucleotide probe (RNA or DNA probe which is prelabeled) and/or antibody probe, salts, detergents, buffers, and blocking agents. The incubation in this solution is carried out at 40°C to 60°C for 5 to 30 minutes.

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In the one-step procedure, regardless of whether the specimen is in suspension or on solid supports, the hybridization procedure is carried out utilizing a single hybridization solution. Optionally, a mild fixative is included in the solution which also fixes the cells. This fixation is accomplished in the same solution and
5 along with the hybridization reaction. The fixative is one which has been found to be optimal for the particular cell type being assayed (eg., there is one optimal fixative for bone marrow and peripheral blood even though this "tissue" contains numerous distinct cell types). The fixative may be a combination of precipitating fixatives (such as alcohols) and cross-linking fixatives (such as aldehydes), with the concentration of
10 the cross-linking fixatives kept very low (less than 10%). The concentration and type of precipitating agent and crosslinking agent may be varied depending upon the probe and the stringency requirements of the probe, as well as the desired temperature of hybridization.

Choice of fixatives and fixation procedures can affect cellular
15 constituents and cellular morphology; such effects can be tissue specific. Preferably, the precipitating fixative has the following characteristics: fixes the cellular constituents through a precipitating action; the effect is reversible, the cellular morphology is maintained, the antigenicity of desired cellular constituents is maintained, the nucleic acids are retained in the appropriate location in the cell, the
20 nucleic acids are not modified in such a way that they become unable to form double or triple stranded hybrids, and cellular constituents are not affected in such a way so as to inhibit the process of nucleic acid hybridization to all resident target sequences. Preferably, fixatives for use in the invention are selected from the group consisting of ethanol, ethanol-acetic acid, methanol, and methanol-acetone. Fixatives most
25 preferable for practicing the one-step procedure include 10-40% ethanol, 10-40% methanol, 10-40% acetone or combinations thereof. These fixatives provide good preservation of cellular morphology and preservation and accessibility of antigens, and high hybridization efficiency. Typically, the solution contains 1-40% ethanol, and 5% formalin.

Fixation of Cells/Tissues in the Two-Step Method

After depositing specimens on solid supports or leaving them in solution, the specimens are fixed. A fixative may be selected from the group consisting of any precipitating agent or cross-linking agent used alone or in combination, and may be aqueous or non-aqueous. The fixative may be selected from the group consisting of formaldehyde solutions, alcohols, salt solutions, mercuric chloride sodium chloride, sodium sulfate, potassium dichromate, potassium phosphate, ammonium bromide, calcium chloride, sodium acetate, lithium chloride, cesium acetate, calcium or magnesium acetate, potassium nitrate, potassium dichromate, sodium chromate, potassium iodide, sodium iodate, sodium thiosulfate, picric acid, acetic acid, paraformaldehyde, sodium hydroxide, acetones, chloroform, glycerin and thymol.

Preferably, the fixative will comprise an agent which fixes the cellular constituents through a precipitating action and has the following characteristics: the effect is reversible, the cellular morphology is maintained, the antigenicity of desired cellular constituents is maintained, the nucleic acids are retained in the appropriate location in the cell, the nucleic acids are not modified in such a way that they become unable to form double or triple stranded hybrids, and cellular constituents are not affected in such a way so as to inhibit the process of nucleic acid hybridization to all resident target sequences. Preferably, fixatives for use in the invention are selected from the group consisting of ethanol, ethanol-acetic acid, methanol, and methanol-acetone which fixatives afford the highest hybridization efficiency with good preservation of cellular morphology.

Simultaneously, the fixative may contain a compound which fixes the cellular components by cross-linking these materials together, for example, glutaraldehyde or formaldehyde. The cross-linking agent is generally more "sticky" and causes the cells and membrane components to be secured or sealed, thus, maintaining the characteristics described above for fixatives. The cross linking agents when used are preferably less than 10% (v/v).

Cross-linking agents, while preserving ultrastructure, often reduce hybridization efficiency by forming networks trapping nucleic acids and antigens and

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rendering them inaccessible to probes and antibodies. Some also covalently modify nucleic acids preventing later hybrid formation. Examples of cross-linking agents include paraformaldehyde, formaldehyde, dimethylsilserimide and ethyldimethylamino-propylcarbodiimide.

5 Storage of Cells/Tissues

After fixation, microscope slides and other solid supports containing specimens may be stored air dried at room temperature for up to six months, in cold (4°C) 70% ethanol in water for 6-12 months. If specimens are handled under RNase free conditions, they can be dehydrated in graded alcohols and stored for at least 12 months at room temperature.

10 Prehybridization Treatments

According to the present invention no formal prehybridization step is necessary. Blocking nonspecific binding of probe and facilitating probe entry can be accomplished in the hybridization solution. If short hybridizations are to be done (less than 30 minutes) slides may be preheated to hybridization temperature before addition of the hybridization solution.

15 Hybridizations

Nucleic acid hybridization is a process where two or more duplex or triplex mirror images or opposite strands of naturally occurring or synthetic DNA, RNA, oligonucleotides, polynucleotides, or any combination thereof recognize one another and bind together through the formation of some form of either spontaneous or induced chemical bond, usually a hydrogen bond. The degree of binding can be controlled based on the types of nucleic acids coming together, and the extent of "correct" binding as defined by normal nucleic acids coming together, and the extent of "correct" binding as defined by normal chemical rules of bonding and pairing. For example, if the binding of two strands forms 9 out of 10 correct matches along a chain of length 10, the binding is said to be 90% homologous.

Cellular nucleic acid sequences are detected by the process of molecular hybridization. The probe must be "labeled" in some way so to allow "detection" of any complementary cellular nucleic acid sequences present within the individual cells.

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In the present invention, the term "hybridization" also means the binding of an antibody to a target antigen.

In the one-step hybridization procedure, the hybridization cocktail contains a denaturing agent, usually formamide, but other chaotropic agents such as NaI, urea, thiocyanate, guanidine, trichloroacetate, tetramethylamine and perchlorate may also be used. Furthermore, several precipitating and/or cross-linking fixatives also have mild denaturing properties; these properties can be used in conjunction with the primary denaturant in either an additive or synergistic fashion. The hybridization cocktail may be constructed to preferentially allow only the formation of RNA-RNA or RNA-DNA hybrids. This is accomplished by adjusting the concentration of the denaturing agents along with the concentration of salts (primarily monovalent cations of the Group I series of metals along with the ammonium ion) and along with the temperature of hybridization which is used. This allows for the selective hybridization of probe to either cellular RNA or DNA or both RNA and DNA simultaneously with distinct probes. This further allows the probes to be supplied in a premixed solution which presents the optimal conditions for generating a signal and minimizing noise while simultaneously optimally "fixes" the morphology of the cells/tissues.

Hybridization Solution Components

The hybridization solution may typically comprise a chaotropic denaturing agent, a buffer, a pore-forming agent, a hybrid stabilizing agent. The chaotropic denaturing agents include formamide, urea, thiocyanate, guanidine, trichloroacetate, tetramethylamine, perchlorate, and sodium iodide. Any buffer which maintains pH at least between 7.0 and 8.0 may be utilized.

The pore-forming agent is, for instance, a detergent such as Brij 35, Brij 58, sodium dodecyl sulfate, Tween, CHAPS or Triton X-100. Depending on the location of the target biopolymer, the pore-forming agent is chosen to facilitate probe entry through plasma, nuclear membranes or cellular compartmental structures. For instance, 0.05% Brij 35 or 0.1% Triton X-100 will permit probe entry through the plasma membrane but not the nuclear membrane. Alternatively, sodium deoxycholate will allow probes to traverse the nuclear membrane. Thus, in order to restrict

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hybridization to the cytoplasmic biopolymer targets, nuclear membrane pore-forming agents are avoided. Such selective subcellular localization contributes to the specificity and sensitivity of the assay by eliminating probe hybridization to complementary nuclear sequences when the target biopolymer is located in the cytoplasm. Agents other than detergents, such as fixatives, may serve this function.

Hybrid stabilizing agents such as salts of mono- and divalent cations are included in the hybridization solution to promote formation of hydrogen bonds between complementary sequences of the probe and its target biopolymer. Preferably, sodium chloride at a concentration from 0.15 M to 1 M is used. In order to prevent non-specific binding of nucleic acid probes, nucleic acids unrelated to the target biopolymers are added to the hybridization solution. Representative examples of hybrid stabilizing agents include sodium chloride, lithium chloride and magnesium.

Preparation of Cells for Flow Cytometry

When cells are analyzed by flow cytometry, the entire procedure is performed while the specimen is maintained in solution. Briefly, cells are suspended in PBS and then pelleted by centrifugation. To the cell pellet is added either a fixative or a hybridization solution containing a fixative. After resuspension, cells are incubated as described below. After pelleting, resuspension and washing, cells are resuspended in the mounting medium and analyzed on a flow cytometer.

Types of Probes

A probe is defined as genetic material DNA, RNA, or oligonucleotides or polynucleotides comprised of DNA or RNA and antibodies. The DNA or RNA may be composed of the bases adenosine, uridine, thymidine, guanine, cytosine, or any natural or artificial chemical derivatives thereof. The probe is capable of duplex or triplex hybrid formation by binding to a complementary or mirror image target cellular genetic sequence. Binding occurs through one or more types of chemical bonds, usually through hydrogen bond formation. The extent of binding is referred to as the amount of mismatch allowed in the binding or hybridization process. The extent of binding of the probe to the target naturally occurring or synthetically produced cellular sequences also relates to the degree of complementarity to the target sequences. The size of the probe is adjusted to be of such size that it forms stable

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hybrids at the desired level of mismatch; typically, to detect a single base mismatch requires a probe of approximately 12-50 bases. Larger probes (from 50 bases up to tens of thousands of bases) are more often used when the level of mismatch is measured in terms of overall percentage of similarity of the probe to the target genetic sequence. The size of the probe may also be varied to allow or prevent the probe from entering or binding to various regions of the genetic material or of the cell. Similarly, the type of probe (for example, using RNA versus DNA) may accomplish these objectives. The size of the probe also affects the rate of probe diffusion, probability of finding a cellular target match, etc. Typically, double-stranded DNA (dsDNA), single-stranded DNA (ssDNA) or RNA probes are used in a hybridization reaction when nucleotide sequences are the target.

Preparation of Probes.

RNA or DNA probes useful in the present invention may be prepared according to methods known to those of skill in the art or may be obtained from any commercial source. RNA probes may be prepared by the methods described by Green et al. (1981) Cell 32:681. DNA probes may be prepared by methods known to those of skill in the art such as described by Rigby et al. (1977) J. Mol. Biol. 113:237. Synthetic oligonucleotide probes may be prepared as described by Wallace, et al (1979) Nucleic Acids Research 6:3543 or as recommended by the suppliers (e.g., ABI) of the nucleic acid synthesizers. The probes useful in the present invention may be prepared according to the methods disclosed in copending patent applications, U.S. Serial No. 784,690, filed October 28, 1991, and Serial No. 668,751, filed March 13, 1991.

Nucleic acid probes can be prepared by a variety of methods known to those of skill in the art. Purified double-stranded sequences of DNA (dsDNA) can be labeled intact by the process of nick translation or random primer extension. The ability of double-stranded probes to hybridize to nucleic acids immobilized within cells is compromised by the ability of the complementary strands to hybridize to each other in solution prior to hybridization with the cellular nucleic acids. Single-stranded DNA (ssDNA) probes do not suffer this limitation and may be produced by the synthesis of oligonucleotides, by the use of the single-stranded phage M13 or plasmid derivatives

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of this phage, or by reverse transcription of a purified RNA template. The use of single-stranded RNA (ssRNA) probes in hybridization reactions potentially provides greater signal-to-noise ratios than the use of either double or single-stranded DNA probes. Regardless of whether a dsDNA, a ssDNA, or a ssRNA triplex or circular probe is used in the hybridization reaction, there must be some means of detecting hybrid formation. The means of detecting hybrid formation utilizes a probe "labeled" with some type of detectable label.

Antibody probes are known to those skilled in the art. The term "antibody probe" means an antibody that is specific for and binds to any target antigen. Such a target antigen may be a peptide, protein, carbohydrate or any other biopolymer to which an antibody will bind with specificity.

Preparation of Antibody Probe

Antibody probes specific for antigens such as viruses or specific determinants thereof, peptides and proteins derived from a variety of sources, carbohydrate moieties and a wide variety of biopolymers are known to those of skill in the art. The methods for preparation of such antibodies are also known to those of skill in the art.

Briefly, polyclonal antibodies may be prepared by immunization of an animal host with an antigen. Preferably, the antigen is administered to the host subcutaneously at weekly intervals followed by a booster dose one month after the final week dose. Subsequently, the serum is harvested, antibodies precipitated from the serum and detectably labeled by techniques known to those of skill in the art.

Monoclonal antibodies may be prepared according to any of the methods known to those in the art. Fusion between myeloma cells and spleen cells from immunized donors has been shown to be a successful method of producing continuous cell lines of genetically stable hybridoma cells capable of producing large amounts of monoclonal antibodies against target antigens such as, for instance, tumors and viruses. Monoclonal antibodies may be prepared, for instance, by the method described in U.S. Patent No. 4,172,124 to Koprowski, et al. or according to U.S. Patent No. 4,196,265 to Koprowski, et al. Procedures for labeling antibodies are known to those skilled in the art.

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Detection Systems

Probes may be detectably labeled prior to addition to the hybridization solution. Alternatively, a detectable label may be selected and added after hybridization is completed and binds to the hybridization product. Probes may be
5 labeled with any detectable group for use in practicing the invention. Such detectable group can be any material having a detectable physical or chemical property. Such detectable labels have been well-developed in the field of immunoassays and in general most any label useful in such methods can be applied to the present invention. Particularly useful are enzymatically active groups, such as enzymes (see Clin. Chem., 22:1243 (1976)); enzyme substrates (see British Pat. Spec. 1,548,741),
10 coenzymes (see U.S. Patents Nos. 4,230,797 and 4,238,565); enzyme inhibitors (see U.S. Patent No. 4,134,792); fluorescers (see Clin. Chem., 25:353 (1979); chromophores; luminescers such as chemiluminescers and bioluminescers (see Clin. Chem., 25:512 (1979)); specifically bindable ligands; and proximal interacting pairs;
15 and radioisotopes such as ^3H , ^{35}S , ^{32}P , ^{125}I and ^{14}C .

Biotin labeled nucleotides can be incorporated into DNA or RNA by nick translation, enzymatic, or chemical means. The biotinylated probes are detected after hybridization using avidin/streptavidin, fluorescent, enzymatic or colloidal gold conjugates. Nucleic acids may also be labeled with other fluorescent compounds, with
20 immunodetectable fluorescent derivatives or with biotin analogues. Nucleic acids cross-linked to radioactive or fluorescent histone HI, enzymes (alkaline phosphatase and peroxidases), or single-stranded binding (ssB) protein also may be used. To increase the sensitivity of detecting the colloidal gold or peroxidase products, a number of enhancement or amplification procedures using silver solutions may be
25 used.

An indirect fluorescent immunocytochemical procedure also may be utilized (Rudkin and Stollar (1977) Nature 265: 472; Van Prooijen, et al. (1982) Exp.Cell.Res. 141: 397). In that procedure, polyclonal antibodies were raised against RNA-DNA hybrids by injecting animals with poly(Ra)-poly(dT). DNA probes were
30 hybridized to cells *in situ* and hybrids were detected by incubation with the antibody to RNA-DNA hybrids.

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Probe Size and Concentration

The length of a probe affects its diffusion rate, the rate of hybrid formation, and the stability of hybrids. According to the present invention, small probes (15-100 bases) yield the most sensitive, rapid and stable system. A mixture of short probes (15-100 bases) are prepared which span the entire length of the target biopolymer to be detected. For example, if the target biopolymer were 1000 bases long, about 40 "different" probes of 25 bases would be used in the hybrid solution to completely cover all regions of the target biopolymer.

The concentration of the probe affects several parameters of the *in situ* hybridization reaction. High concentrations are used to increase diffusion, to reduce the time of the hybridization reaction, and to saturate the available cellular sequences. To achieve rapid reaction rates while maintaining high signal-to-noise ratios, probe concentrations of 0.01 to 100 $\mu\text{g/ml}$ of hybridization solution are preferable. Most preferable is use of probes at a concentration of 2.5 $\mu\text{g/ml}$.

Hybridization Solution and Temperature

In a preferred embodiment, the hybridization solution of the one-step *in situ* method consists of 25% formamide, 5X SSC, 15 X Ficoll/PVP, .4M guanidinium isothiocyanate, about 50 mM sodium phosphate (Ph 7.4), 50 mM DTT, about 1 mg/ml salmon sperm DNA, 5% Triton X-100, 50 mM EDTA and 21% PEG. A synthetic oligonucleotide probe is added to this solution. The probe may be at least 15-20 bases, preferably, about 25 bases, and labeled with PhotobiotinTM. The most preferable, optimal temperature of hybridization is 40°-45°C. However, temperatures ranging from 15°C to 80°C may be used.

In the one-step and two-step methods, the probe in the hybridization cocktail may be labeled before the hybridization reaction. The label may be one of the many types described above. If the probe is labeled with PhotobiotinTM, the hybrids may be detected by use of a Streptavidin/Avidin (S/A) conjugated to a fluorescent molecule such as FITC, rhodamine, Texas RedTM; to S/A conjugated to an enzyme; or to S/A labeled with a heavy metal such as colloidal gold. Specifically, a solution containing the streptavidin conjugate is added directly to the hybridization cocktail over the cells after the end of the hybridization reaction. The cells are

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incubated in this solution for 5 to 30 minutes at 45°C. Longer times of hybridization, however, may be used along with both higher or lower temperatures. The time of hybridization reaction will vary depending on the composition of the hybridization cocktail containing the fixative (type and concentrations of precipitating agents and/or cross-linking agents), buffering agents, pore forming agents, denaturing agents and hybrid stabilizing agents. Similarly, the temperature may be varied as described.

Alternatively, the probes may be directly labeled with the fluorescent dye or molecules such as Pontamine Sky Blue™ by incubating the nucleic acid probe and dye together (1:10 weight:weight proportions) and allowing the dye to bind/intercalate. The probe is then precipitated out of the dye solution and the excess unbound dye is removed by repeated washing with 70% ethanol. Probes also are labeled directly and covalently by incubation of double stranded molecules (RNA-RNA, RNA-DNA, or DNA-DNA) with labels which will covalently bind to nucleic acids. After incubation conditions under which the reaction will take place, the strands are separated and each separate strand is used as a probe. The concentration of the probe in the solution is typically 2.5 µg/ml although a range of 0.01-100 µg/ml is useful. The probe concentration will affect the reaction kinetics and may affect the sensitivity of the assay along with the signal-to-noise ratio.

If the probe is labeled directly with an enzymatic label or is detected using an enzymatic or secondary detectable system, then this reaction may be carried out before or after any wash steps. Following the incubation of the specimen with the appropriate buffer for the enzyme, the slide is incubated with the substrates for the enzyme under conditions specified by the manufacturer or supplier of the enzyme.

Cells may be deposited onto slides or centrifuged into a pellet following the fixation/hybridization/ detection reaction(s). Next, the unbound probe is washed away from the cells by one wash step using a solution of 0.1 x SSC with 0.1% Triton X-100™. A total of 1-200 ml of wash solution may be used per microscope slide (i.e., per about 100,000 separated cells or per tissue section of about 1 square centimeter). The concentration and type of the hybrid stabilizing/denaturing agents and pore forming agents may be varied depending on the type of cells, the type of probe and the acceptable level of mismatch of the hybrid.

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Results obtained using the one-step or two-step method.

When cells are deposited onto slides, results are visualized manually on a fluorescent microscope when direct or indirectly labeled fluorescent probes are utilized. Alternatively, the results may be automatically analyzed on a fluorescence-based image analysis system.

When cells are maintained in solution, results may be obtained using a flow cytometer to record the amount of fluorescence per cell, which represents the amount of hybrid per cell. Alternatively, the total signal within a cellular sample may be determined using a device such as a liquid scintillation counter (for radioactivity) or a chemiluminescent/fluorescent microtiter plate reader for these labels.

Speed, Sensitivity and Quantification of *In situ* Hybridizations

In the one-step embodiment, the present invention requires as little as 5 minutes to complete with a sensitivity of as few as 1 molecule of a cellular biopolymer per cell. The speed and sensitivity result from the combination of at least four factors: 1) cellular constituents are not irreversibly precipitated or fixed onto the nucleic acids, 2) if fixed, the fixation was optimized for the particular tissue used 3) the kinetics of the reaction proceed more rapidly at high probe concentrations and at elevated temperatures, and 4) probes were constructed to facilitate rapid entry and exit through cellular membranes and cellular components.

The number of copies of mRNA or DNA per cell can be estimated from the number of grains over cells when radioactive probes are used. With fluorescent or enzymatic detections a relative estimate of fluorescence or precipitated colored products allows estimation of mRNA or DNA copy number. Usually, the approximation of copy number is easier after manual photography, film processing and comparisons of photographic prints. The quantitation of radioactive or fluorescent signals obtained after *in situ* hybridizations may be automated by use of an image analysis system, such as the Meridian ACAS 570 workstation.

The sensitivity of the *in situ* hybridization techniques of the present invention permit the visual and photographic detection of a single copy of a genetic sequence present within a single cell.

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For example and not by way of limitation, using a single fluorophor on each probe, with each probe being about 30 bases in length, a genetic sequence of approximately 6,000 bases can be reliably detected by viewing the results through a standard fluorescent microscope. When four fluorophors are attached to a single probe, the presence or absence of a particular genetic sequence of approximately 1500 bases can be reliably detected, using the period of incubation taught herein. Moreover, when using probes for corresponding sequences from both the "sense" and the "anti-sense" strands of a two-stranded nucleic acid, one can detect the presence or absence of a sequence as short as approximately 750 base pairs using such periods of incubation described herein. Alternatively, one may detect as few as 75 to 150 base pairs using an image analysis system.

Simultaneous Detection of Three mRNAs

Both the manual and one-step *in situ* hybridization procedures allow simultaneous detection of different substances (mRNAs, DNAs and proteins) within the same cells. This may be accomplished in one of two ways. First, multiple probes each containing a unique label (for example, fluorescent tags "A", "B" and "C" which each emit light at a different detectable wave length) are all added together in the hybridization solutions. Alternatively, a hybridization and detection reaction may be carried out with one probe and label, residual unreacted probe and label washed away, and another hybridization reaction is carried out. This process is repeated as many times as desired. Alternatively, probes A, B and C all contain the same tag.

When DNA and RNA were both detected, the selection of the type of probe became important. When the cellular target biopolymer is RNA, an anti-sense, single stranded DNA probe was used in the assay. If the cellular target DNA is the biopolymer to be detected, a sense-strand, single-stranded RNA probe would be used in the assay. This probe selection, and the selection and concentration of components of the fixation/hybridization solution would allow only RNA-DNA hybrids to be formed. Therefore, the probe could only bind to the desired target cellular biopolymer; other nucleic acids would inherently be prevented from interfering with the reaction assay.

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The one-step *in situ* hybridization technique for the detection of DNA or mRNA may be provided as a kit. Such a kit includes the following:

1. A solution containing a fixation/hybridization cocktail and one or more labeled probes. Preferably, this solution will contain 50 mM guanidinium isothiocyanate, 25-40% formamide, 21% PEG, 0.4 M DTT, 15X Ficoll/PVP, 50 mM EDTA, 1 mg/ml salmon sperm DNA, 50 mM Tris-acetate (Ph 7-8), about 5% Triton X-100, and about .06 $\mu\text{g}/\mu\text{l}$ of a synthetic oligonucleotide probe directly labeled with a reporter molecule. This solution and the probes would have measurable predefined and identified characteristics and reactivities with cells and target sequences.
2. Means and instructions for performing the said *in situ* hybridization reaction of the present invention.

Alternatively, the kit may also include:

1. A second detectable reporter system which would react with the probe or the probe-target hybrid.
2. Concentrated stock solution(s) to be used directly or to be diluted sufficiently to form wash solution(s).
3. Any mechanical components which may be necessary or useful to practice the present invention such as a solid support (e.g. a microscope slide), an apparatus to affix cells to said support, or a device to assist with any incubations or washings of the specimens.
4. A photographic film or emulsion with which to record results of assays carried out with the present invention.

Another version of this kit may include a solution of probes encapsulated in liposomes or microspheres.

The following examples are offered by way of illustration and are not intended to limit the invention in any manner. In all examples, all percentages are by weight if for solids and by volume if for liquids, and all temperatures are in degrees Celsius unless otherwise noted. All literature citations are expressly incorporated by reference.

EXAMPLE 1***In situ* Hybridization Procedure Using DNA Probes To Detect HPV On A Previously Stained Papanicolaou Smear**

5 Cervical cells obtained using a cervical brush were placed in the transport medium. Upon arrival in the laboratory, the cells were vortexed for 1-3 seconds, then spun at 1500 rpm for 10 minutes. The supernatant was discarded and the cells resuspended in 1 ml of the transport medium. The cells were spotted directly on organosilane treated slides at the desired density. Cells were then stained using the standard papanicolaou staining procedure.

10 Dip slides 5 times in H₂O. Stain 10 seconds in Gills formulation #2; followed by 10 dips in deionized water. Dip 10 times in 95% ETOH. Stain 1 minute in OG-50. Dip 10 times in 95% ETOH. Stain 2 minutes in EA-50. Destain in 95% ETOH; 10 dips. On a standard frosted end slide add 50 ul GEL/mount (Biomedica Corp., Foster City, CA.- Catalog M01). Cover with a 24 by 50 mm coverslip and let set for 10 minutes.

15 Prior to *in situ* hybridization, the slides were soaked in DDH₂O until the coverslips fell off, usually about 10 minutes. The slides were soaked an additional 5 minutes in fresh H₂O. Then dehydrated in graded (50%, 70%, & 95%) ethanol solutions.

20 **Preparation Of Probes**

The positive control probe consisted of a human alpha-centromeric repeat DNA, known to hybridize to all human chromosomes. The negative probe, designated NR, was derived from the nitrogen reductase gene found in bacteria and was known to not hybridize to nucleic acid within eukaryotic cells. The sequences for HPV type 16 and HPV type 18 were obtained from the published sequences and were accessed via the Genetic Sequence Data Bank, GenBank, version 69.0.

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	Probe	GenBank	Fluorescent	Molecular
	Designation	Locus Name	Label	Probes, Inc. Cat. #
5	HPV16	PPH16	Rhodamine Derivative	L-20
	HPV18	PPH18	Rhodamine Derivative	L-20

Five hundred separate probes (250 for type 16 and 250 for type 18) were designed as 25-bases in length and were synthesized.

10 Several 25-base synthetic oligonucleotide probes were prepared from each of the DNA sequences listed below.

TABLE 1

	Probe	Chromosome	GenBank	Fluorescent
	Designation	Detected	Locus Name	Label
15	Alpha-centromeric repeat	X	HUMSATA	Fluorescein
	Alpha-centromeric repeat	Y	HUMSATB	Fluorescein

Probe Synthesis and Labeling

The oligodeoxynucleotides were synthesized (Applied Biosystems DNA Synthesizer Model 380 B using the recommended A.B.I. reagents), and in the last stage

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an aminohexyl phosphate linker was attached to the 5' end. The 5'-aminohexyl oligodeoxynucleotides were then coupled to a fluorescent rhodamine derivative (Cat. # L-20) from Molecular Probes, Inc. and purified by Waters HPLC using a baseline 810 chromatography work station.

5 Hybridization

For the hybridization procedure, 50 μ l of an hybridization cocktail consisting of 22% PEG, 30% formamide, 5X SSC, .3 mg/ml salmon sperm DNA, 15X Ficoll/PVP, .4M guanidinium isothiocyanate, 50mM DTT, 5% Triton X-100, 50mM EDTA, 7.5% Tween 20, 50mM Na₂PO₄, and probe at a concentration of .02 μ g/ μ l was added to the slide. A coverslip was applied and the slide was heated to 95°C for 5 minutes, allowed to cool to 42°C and incubated for 25 minutes at that temperature.

10 Washing

Post-hybridization, the slides were placed in a coplin jar to which was added 100 ml of a wash solution, consisting of .1X SSC and .4M guanidinium isothiocyanate and .1% Triton X-100. The solution was agitated until the coverslip fell off and held in this solution for 4 minutes. This wash solution was removed and a second wash solution, consisting of .1X SSC and .1% Triton X100 was added. This solution was agitated for 1 minute, poured off and the wash was repeated 5 times. Following the washes, 8 μ l of antifade/Hoechst counterstain was added. The slide was coverslipped and viewed under the fluorescent microscope.

20 Fluorescence Detection

Photomicrographs were taken on an Olympus BH10 microscope with fluorescence capabilities, using Kodak Ektachrome EES-135 (PS 800/1600) film, exposed, and push processed at 1600 ASA. A 50-second exposure time was consistently used, so that direct comparisons could be made between all photomicrographs taken.

25 Results

Figure 1 demonstrates a typical result when the HPV probes (labeled with Rhodamine) and the positive control probe (labeled with FITC) are added together in the same hybridization cocktail. The nucleus of the smaller cell has stained positive for the positive control probe (in color this is green) while the larger cell nucleus is positive for HPV (in color this is bright yellow).

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The procedure of Example may be modified as follows:

1) Four hundred and sixteen (400) separate probes (200 for type 16 and 200 for type 18) each designed as 30-bases in length, are synthesized. However, in addition to making probes corresponding to those 400 separate oligonucleotides that together comprise probes for one strand of each of the two HPV targets, one also makes 400 additional oligonucleotide probes for the second strands of both of the two HPV targets. The probes for the first strand will be "out of phase" relative to the second strand probes as regards how they map on a map of the HPV genome. As a result, one half (15 nucleotides) of each first strand probe will be complementary (in nucleotide sequence) to one half of one second strand probe and the other half (15 nucleotides) of that first strand probe will be complementary to a portion of another second strand probe. Staggering of the probes means that, because of the shortness of the overlap (15 nucleotides), probes of the first strand will not hybridize significantly to probes of the second strand. On the other hand, about twice as much hybridization is detected as compared to the situation where only probes corresponding to one strand are used.

2) Probes are made as phosphorothioate oligonucleotides, each 30-mer having four sulfur atoms, using an Applied Biosystem (ABI) DNA Synthesizer, Model 380B and the recommended ABI reagents. The sulfur atoms are located as follows: one is at the extreme 5' end of the probe, a second is between the 7th and 8th nucleosides (counting from the 5' end), the third is between the 22nd and 23rd nucleosides, and the fourth is between the 29th and 30th nucleosides. The sulfur atoms of the polysulfurized oligonucleotides are then coupled to a fluorescent dye, iodoacetamido-fluorescein, as follows (smaller amounts can be synthesized by adjusting the volumes): 200 μ g of dried oligonucleotide is dissolved in 100 μ l of 250 mM Tris buffer, pH 7.4 to form a first solution. Then one mg of iodoacetamido-fluorescein is combined with 100 μ l of dry dimethylformamide (i.e., 100 percent DMF) in a second solution. The two solutions are mixed together and shaken overnight. After the overnight incubation, the labeled oligonucleotide is precipitated with ethanol and 3M sodium acetate. This crude material is then loaded on to a PD-10 column to remove free dye. The desired fractions are then collected. The liquid phase is then removed under vacuum. The crude material is then purified with HPLC (high performance liquid chromatography).

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3) Negative and positive control probes are constructed in analogy to steps (1) and (2).

4) The hybridization cocktail is modified as follows: 1.5% PEG is used instead of 21% PEG, 30% formamide is used instead of 21% formamide, 10% DMSO (10% v/v) is included, and 5% (v/v) of vitamin E is included. Also instead of adding 50 μ l of the hybridization cocktail to the slide, 40 μ l of the cocktail is added to 5 μ l of squalene plus 5 μ l of pyrrolidinone and the combined 50 μ l is added to the slide. It can be useful to add 5 μ l of 1 M (1 molar) DTT and 5 μ l of Proteinase K (1 mg/ml) solution per 100 μ l of hybridization cocktail and run the hybridization reaction at, for example, 42°C for 5 minutes, then at 95°C for 5 minutes. It can also be useful to add about 0.50% or 0.10% aurintricarboxylic acid in the hybridization cocktail.

5) Instead of adding 8 μ l of antifade/Hoechst to the slide, 8 μ l of the following solution is added: 9 volumes of solution A plus one volume of solution B where solution A is 0.01% 1,4 diphenylamine (antifade) plus nuclear stain Hoechst (#33258; 1 μ g/ml) plus 0.0025% Evans Blue in 50% (v/v) glycerol plus 50% (v/v) 1 \times PBS (0.136 M NaCl, 0.003 M KCl, 0.008 M Na₂HPO₄, 0.001 M KH₂PO₄) and solution B is dodecyl alcohol.

EXAMPLE 2

In situ Hybridization Procedure Using DNA Probes To Detect *Chlamydia trachomatis* and *Neisseria gonorrhea* DNA or mRNA On A Previously Stained Papanicolaou Smear
Preparation of Cells

Cervical cells obtained using a cervical brush are placed in the transport medium. Upon arrival in the laboratory the cells are vortexed for 1-3 seconds, then spun at 1500 rpm for 10 minutes. The supernatant is discarded and the cells are resuspended in 1 ml of the transport medium. The cells are spotted directly on organosilane treated slides at the desired density. Cells are then stained using the standard papanicolaou staining procedure as described in Example 1.

Preparation of Probes:

The positive control probe is as described in Example 1.

The sequence for a 7.4 kb plasmid that is a common component of the CT genome and occurs in approximately 10 copies per genome is accessed via the Genetic

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Sequence Data Bank, GenBank, version 69.0. This entire 7.4 kb sequence is cut into 296 separate oligonucleotide probes consisting of 25 bases each. These 296 oligomers are synthesized and labeled as described below.

5 For GC, three repetitive DNA elements are accessed via the Genetic Sequence Data Bank, GenBank, version 69.0.

These sequences are cut into separate oligonucleotide probes consisting of 25 bases each and are synthesized and labeled as described below.

TABLE 2

10	Probe	GenBank
	Designation	Locus Name
	C.T.	CHTP
	G.C.	NGORPTA
		NGORPTB
15		NGORPTC

TABLE 3

20	Fluorescent	Molecular Probes
	Label	Cat. #
	Fluorescein	F-143
	Rhodamine Derivative	L-20
	Texas Red	T-1905
25	Coumarin Derivative	D-1412

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Probe Synthesis and Labeling

The oligodeoxynucleotides are synthesized (Applied Biosystems DNA Synthesizer Model 380 B using the recommended A.B.I. reagents), and in the last stage an aminohexyl phosphate linker is attached to the 5' end. The 5'-aminohexyl oligodeoxynucleotides are then coupled to a fluorochrome from Molecular Probes, Inc. and purified by Waters HPLC using a baseline 810 chromatography work station.

For simultaneous detection of both organisms GC and CT probes can be labeled with different fluorescent moieties. For example, GC can be labeled with fluorescein while CT can be labeled with a rhodamine derivative. Alternative fluorochromes, with catalogue numbers are listed above.

Hybridization

For the hybridization procedure, 50 μ l an hybridization cocktail consisting of 30% PEG, 30% formamide, 5X SSC, .3 mg/ml salmon sperm DNA, 15X Ficoll/PVP, .4M guanidinium isothiocyanate, 50mM DTT, 5% Triton X-100, 50mM EDTA, 50mM Na_2PO_4 , and probe at a concentration of .02 $\mu\text{g}/\mu\text{l}$ is added to the slide. A coverslip is applied and when DNA is the target, the slide is heated to 95°C for 5 minutes, allowed to cool to 42°C and incubated for 25 minutes at that temperature. If mRNA is the target, the 95°C heating step is omitted.

Washing

Post-hybridization, the slides are placed in a coplin jar to which is added 100 ml of a wash solution, consisting of .1X SSC and .4M guanidinium isothiocyanate and .1% Triton X-100. The solution is agitated until the coverslips fall off and held in this solution for 4 minutes. This wash solution is removed and a second wash solution, consisting of .1X SSC and .1% Triton X-100 is added. This solution is agitated for 1 minute and poured off and this last wash is repeated 5 times. Following the washes, 25 μ l of antifade/Hoechst counterstain is added. The slide is coverslipped and is viewed under the fluorescent microscope.

Fluorescence Detection

Photomicrographs are taken on an Olympus BH10 microscope with fluorescence capabilities, using Kodak Ektachrome EES-135 (PS 800/1600) film, exposed, and push processed at 1600 ASA. A 50-second exposure time is consistently

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used so that direct comparisons could be made between all photomicrographs taken. Alternatively, commercially available low light monochrome and color cameras allow the operator to both visualize the results on a TV screen and make a permanent record in the form of a color print.

- 5 The CT and GC organisms stain brightly positive when the CT and GC specific probes are used, respectively.

EXAMPLE 3

In situ Hybridization Procedure Using DNA Probes To Detect Herpesvirus II DNA or mRNA On A Previously Stained Papanicolaou Smear

10 Preparation of Cells

Previously stained cervical cells are prepared as in Example 1.

Positive Control Probe:

The positive control probe is as described in Example 1.

Herpesvirus II (HSV-II) Specific Sequences:

- 15 The sequence for HSV-II is accessed via the Genetic Sequence Data Bank, GenBank, version 69.0. The sequences for the immediate early and early genes for HSV-II are cut into separate oligonucleotide probes consisting of 25 bases each. These oligomers are synthesized and labeled as described in Example 1.

Hybridization, Washing and Fluorescence Detection

- 20 Hybridization, washing and detection are carried out as described in Example 1.

HSV-II containing cells stain brightly positive when the HSV-II probe is used

EXAMPLE 4

- 25 *In situ* Procedure Using DNA Probes To Detect HPV In Cells Previously Stained With A Papanicolaou Stain And Demonstrating Simultaneous Detection And Differentiation of HPV16 From HPV18

Preparation of Cells

- 30 The C-33A (ATCC HTB #31), Ca Ski (ATCC CRL #1550) and Hela Cells were grown, trypsonized, mixed at a 1:1:1 ratio, and spotted directly on organosilane

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treated slides at the desired density. Cells were then stained using the standard papanicolaou staining procedure and prepared for *in situ* hybridization as outlined in Example 1.

Preparation of Probes

5 The positive control probe was as described in Example 1.

The sequences for HPV type 16 and HPV type 18 were obtained from the published sequences and were accessed via the Genetic Sequence Data Bank, GenBank, version 69.0 as described in Example 1.

Probe Synthesis and Labelling

10 The oligodeoxynucleotides were synthesized and labeled as described in Example 1. Fluorescein (Cat. # F-143) was attached to the HPV16 probe and a rhodamine derivative (L-20) to the HPV18 probe.

Hybridization and Washing and Fluorescent Detection

15 As in Example 1, the hybridization, washing and fluorescent detection steps were performed.

Results

20 Figure 2 depicts the three different cell types in a single photograph. The FITC labeled HPV-16 probes resulted in a positive signal (dots of light in the nucleus) in CaSki cells in the upper left corner of the "2A" photo while the rhodamine labeled HPV-18 probe resulted in a positive signal (dots of light in the nucleus) in the lower right corner of this same "2A" photo. The cell in the center of the photo is a C-33A cell and is appropriately negative. "2B" is a photo of the Hoechst stained nuclei of all three cells. This demonstrates the ability of this assay system to simultaneously detect and differentiate the two HPV types 16 and 18, within cells previously stained.

25

EXAMPLE 5

In situ Procedure Using DNA Probes To Detect A Single Copy Of HPV On Cells Previously Stained With A Papanicolaou Stain

Preparation of Cells

30 The cell lines C-33A (ATCC HTB #31), Caski (ATCC CRL #1550), and SiHa (ATCC HTB #35) are grown, trypsonized and spotted directly on organosilane treated slides at the desired density. Cells are then stained using the standard

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papanicolaou staining procedure and prepared for *in situ* hybridization as described above in Example 1.

Preparation Of Probes

5 The positive control probe and the sequences for HPV 16 are prepared as described in Example 1

Probe Synthesis and Labeling

The oligodeoxynucleotide probes are synthesized and labeled as described in Example 1.

Hybridization, Washing and Fluorescent Detection

10 As described in Example 1, the hybridization, washing and detection steps are performed.

Since there is a single integrated DNA copy of HPV type 16 in the SiHa cells, there is a single point of light in the SiHa cells. The CaSki cells have several points of light while the C-33A cells have none.

15

EXAMPLE 6

In situ Procedure Using DNA Probes To Detect HPV In Cervical Tissue Sections Previously Stained With Hematoxylin And Eosin (H&E)

Preparation of Tissue

20 Formaldehyde fixed tissue that has been embedded in paraffin can be treated in the following fashion to remove the coverslip and stain with H & E:

1. Xylene, three changes 2 minutes each
2. Absolute alcohol 10 dips
3. 95% alcohol, two changes 10 dips each
4. Tap water rinse until water runs off evenly.
- 25 5. Hematoxylin - Delafield's, Ehrlich's, or Harris' without acetic acid 10 to 15 minutes
6. Tap water, two changes 10 dips each.
7. 1% Hydrochloric acid in 70% alcohol 5 to 10 dips
8. Running water wash well.
- 30 9. Ammonia water, 0.24% or lithium carbonate, 0.5% 30 seconds.
10. Tap water, two changes 10 dips each.

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11. Eosin 10 to 20 dips.
or eosin-phloxine1 to 3 minutes.
12. 95 % alcohol, two changes 10 dips each.
13. Absolute alcohol, three changes. . . . 10 dips each.
- 5 14. Xylene, three changes. . . .10 dips each.
15. 95 % alcohol, two changes10 dips each.
16. 70 % alcohol, two changes10 dips each.
17. 50 % alcohol, two changes10 dips each.
18. DDH₂O, two changes10 dips each.
- 10 19. Proceed with hybridization as described below.

Preparation Of Probes

The sequences for HPV type 16 and HPV type 18 were obtained as described in Example 1.

Probe Synthesis and Labelling

- 15 The oligodeoxynucleotide probes were synthesized and labeled as described in Example 1.

Hybridization, Washing and Fluorescent Detection

As described in Example 1, the hybridization, washing and detection steps were performed.

20 Results

- Figure 3 demonstrates the detection of HPV types 16 and 18 in cervical tissue previously stained with Hematoxylin and Eosin (H & E). Panel 3A is a photo (magnification = 10X) of the H & E stained cervical tissue, prior to hybridization. Panel 3B shows a photo (magnification = 40X) of the Hoechst stained nuclei while panel 3C shows a photo (magnification 40X) of HPV positively stained cells following hybridization with the HPV containing cocktail. While all cells are brightly positive with the Hoechst stain, only a few cells are brightly positive for HPV. Negative control probes were appropriately negative.
- 25

EXAMPLE 7

- 30 *In situ* Procedure Using DNA Probes To Detect The Multidrug Resistance Gene and ErbB Gene In a Tissue Specimen Previously Stained With Hematoxylin and Eosin

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Preparation of Cells

Tissue obtained following a biopsy of the human cervix or breast, fixed in formaldehyde, and stained with H & E is analyzed.

Preparation Of Probes

5 The positive control probe consists of the probes described in Example 1.

The sequences for c-erb-B-2 and MDR1 are obtained from published sequences and are accessed via GenBank version 69.0.

TABLE 4

10	Probe	GenBank	Fluorescent
	Designation	Locus Number	Label
<hr/>			
	c-erb-B-2	HUMERB2R	Any fluorochrome
	MDR1	HUMMDR1	listed in Example 2
15	<hr/>		

Probe Synthesis and Labeling

The oligodeoxynucleotides are designed and synthesized and labeled as described in Example 1.

Hybridization and Washing and Fluorescent Detection

20 As in Example 1, the hybridization, washing and fluorescent detection steps are performed.

Cells containing several copies of the c-erb-B2 or MDR1 gene have a nuclear and/or cytoplasmic stain that is significantly greater than the normal controls, i.e., cell with a single DNA copy of the gene. In addition, the amount of signal can be
25 quantitated with any number of image analysis systems, e.g., ACAS 570 by Meridian Instructions, Okemos, Michigan.

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EXAMPLE 8

In situ Procedure Using DNA Probes to Detect chromosomal DNA on a Specimen Previously Stained by the Diff-Quick Method

Preparation of Cells

5 Cervical cells obtained using a cervical brush were placed in the transport medium. Upon arrival in the laboratory the cells were vortexed (1-3 seconds), then spun at 1500 rpm for 10 minutes. The supernatant was disregarded and the cells resuspended in 1 ml of the transport medium. The cells were spotted directly on organosilane treated slides at the desired density. Cells were then stained using the Diff-Quick Method,
10 following the recommendations of supplier. Coverslips were removed as described in Example 1.

Preparation of Probes

The positive control probes were synthesized and labeled as described in Example 1.

15 **Hybridization and Washing and Fluorescent Detection**

As in Example 1, the hybridization, washing and fluorescent detection steps were performed.

Results

20 Figure 4 depicts the results obtained in cervical cells stained by the Diff-Quick method (4C is 10X magnification while 4D is 40X) followed by hybridization containing the positive control probes (4A is 10X magnification while 4B is 10X). The bright positive signal is clearly seen in the nucleus of these cells and demonstrates that the chromosomal probes hybridize to DNA within these previously stained cells.

EXAMPLE 9

25 *In Situ Hybridization Procedure Using DNA Probes To Detect Trichomonas vaginalis, Candida species, and Treponema pallidum DNA or mRNA On A Previously Stained Papanicolaou Smear*

Preparation of Cells

30 Cervical cells obtained using a cervical brush are prepared and stained as in Example 1. Scrapings of vagina lesions are applied to clean glass slides in the standard fashion.

Positive Control Probe

The positive control probe is as described in Example 1.

Candida albicans, *Trichomonas vaginalis* and *Treponema pallidum*

5 The sequences for *Candida albicans*, *Trichomonas vaginalis* and *Treponema pallidum* are accessed via a Sequence Data Bank or from published sequences. The sequences are cut into separate oligonucleotide probes consisting of 25 bases each. These oligomers are synthesized and labeled as described in Example 1.

Hybridization and Washing and Fluorescent Detection

10 As in Example 1, the hybridization, washing and detection steps are performed.

The *Candida*, *Trichomonas* and *Treponema* microorganisms stain brightly positive when the specific probes designed to detect them are used respectively.

15 All patents and publications mentioned in this specification are indicative of the levels of those skilled in the art to which the invention pertains. All patents and publications are herein incorporated by reference to the same extent as if each individual publication was specifically and individually indicated to be incorporated by reference.

20 One skilled in the art will readily appreciate that the present invention is well adapted to carry out the objects and obtain the ends and advantages mentioned, as well as those inherent therein. The components, methods, procedures and techniques described herein are presently representative of the preferred embodiments, are intended to be exemplary, and are not intended as limitations on the scope of the present invention. Changes therein and other uses will occur to those skilled in the art which are encompassed within the spirit of the invention and are defined by the scope of the appended claims.

25 What is claimed is:

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Claims

1. A method of detecting the presence of a biopolymer in a previously stained specimen having substantially intact cellular membranes by assaying cellular biopolymers comprising the steps of:

5 fixing said specimen with a fixation medium comprising at least one agent selected from the group consisting of a precipitating agent and a cross linking agent,

 contacting said fixed specimen with a hybridization solution comprising of a denaturing agent, a hybrid stabilizing agent, a buffering agent, a selective
10 membrane pore-forming agent and at least one probe, said contacting being under hybridizing conditions,

 detecting hybrid formation by means of said label.

2. The method of Claim 1, wherein said hybrid formation is selected from the group consisting of duplex and triplex.

15 3. The method of Claim 1, wherein said probe has a nucleotide sequence at least substantially complementary to a specific target nucleotide sequence to be detected.

 4. The method of Claim 3, wherein said target nucleotide sequence is about 75 bases.

 5. The method of Claim 1, wherein said specimen comprises a
20 microorganism.

 6. The method of Claim 5, wherein said microorganism is selected from the group consisting of bacteria, viruses and fungi.

 7. The method of Claim 1, wherein said specimen comprises a eukaryotic cell.

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8. The method of Claim 7, wherein said eukaryotic cell is a human cell.
9. The method of Claim 1, wherein said specimen comprises a cellular gene.
10. The method of Claim 9, wherein said cellular gene is selected from the group consisting of an oncogene, a tumor suppressor gene and a stimulating growth factor.
- 5
11. The method of Claim 10, wherein said oncogene is selected from the group consisting of c-erb-B-2, c-myc, c-myb, and c-ras.
12. The method of Claim 10, wherein said tumor suppressor gene is selected from the group consisting of P-53 and retinoblastoma gene.
- 10
13. The method of Claim 10, wherein said stimulating growth factor is selected from the group consisting of transforming growth factor- α , epidermal growth factor and color stimulating factor-granulocyte/macrophage.
14. The method of Claim 6, wherein said bacteria in said previously stained specimen is selected from the group consisting of *Streptococcus*, *Staphylococcus*,
15 *Clostridium*, *Bacillus*, *Pseudomonas*, *Salmonella*, *Klebsiella*, *Bacteroides*, *Escherichia coli*, *Neisseria gonorrhoea*, and *Chlamydia*.
15. The method of Claim 6, wherein said bacteria is selected from the group consisting of *Chlamydia trachomatous* and *Neisseria gonorrhoea*.
16. The method of Claim 6, wherein said fungi is selected from the group
20 consisting of *Candida*, *Cryptococcus neoformans* and *Blastomyces dermatitides*, *Histoplasma capsulatum*, *Coccidioides immitis* and *Paracoccidioides brasiliensis*.

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17. The method of Claim 1, wherein said previously stained specimen is selected from the group consisting of a cell sample and a tissue sample.

18. The method of Claim 17, wherein said cell sample is selected from the group consisting of cervical cells, bone marrow cells, hepatocytes, cerebrospinal fluid cells, blood cells, oral mucosa, lung cells and skin cells.

19. The method of Claim 17, wherein said tissue sample is selected from the group consisting of lymph node tissue, mammary tissue, cervical tissue, colon tissue, prostate tissue, cardiac tissue and brain tissue.

20. The method of Claim 1, wherein said previously stained specimen was stained with a stain selected from the group consisting of a papanicolaou stain, a Wright stain, a Hematoxylin and Eosin stain and Diff-Quick stain.

21. The method of Claim 6, wherein said virus in said previously stained specimen is selected from the group consisting of human papilloma virus, herpes simplex virus II, hepatitis, human immunodeficiency virus, influenza virus, parainfluenza virus and rota virus.

22. The method of Claim 14, wherein said biopolymer is mRNA.

23. The method of Claim 14, wherein said biopolymer is DNA.

24. The method of Claim 14, wherein said biopolymer is 16S rDNA.

25. The method of Claim 14, wherein said biopolymer is selected from the group consisting of mRNA, DNA, a synthetic biopolymer and 16S rDNA.

26. The method of Claim 14, wherein said biopolymer is a synthetic biopolymer.

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27. The method of Claim 21, wherein said biopolymer is mRNA.
28. The method of Claim 21, wherein said biopolymer is a synthetic biopolymer.
29. The method of Claim 21, wherein said biopolymer is DNA.
- 5 30. The method of Claim 21, wherein said biopolymer is 16S rDNA.
31. The method of Claim 21, wherein said biopolymer is selected from the group consisting of mRNA, DNA, a synthetic biopolymer and 16S rDNA.
32. The method of Claim 14, wherein said specimens are cervical cells.
33. The method of Claim 21, wherein said specimens are cervical cells.
- 10 34. The method of Claim 14, wherein said staining is a papanicolaou stain.
35. The method of Claim 21, wherein said stain is a papanicolaou stain.
36. The method of Claim 1, wherein said label is attached to said probe.
37. The method of Claim 1, wherein said label is added after the hybrid formation is complete.
- 15 38. The method of Claim 1, wherein said label is selected from the group consisting of radioactive labels, fluorescers, chemilumescers, and enzyme labels.
39. The method of Claim 1, wherein said label is a conjugate of avidin and streptavidin.

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40. The method of Claim 1, wherein said fixative is selected from the group consisting of ethanol, methanol, acetone and formaldehyde.

5 41. The method of Claim 1, wherein said cross-linking agent is selected from the group consisting of paraformaldehyde, formaldehyde, dimethylsilserimide and ethyldimethylamino-propylcarbodiimide.

42. The method of Claim 1, wherein said denaturing agent is selected from the group consisting of formamide, urea, sodium iodide, thiocyanate, guanidine, perchlorate, trichloroacetate and tetramethylamine.

10 43. The method of Claim 1, wherein said hybrid stabilizing agent is selected from the group consisting of sodium chloride, lithium chloride, magnesium chloride and ferric sulfate.

44. The method of Claim 1, wherein said pore forming agent is selected from the group consisting of Brij 35, Tween, Brij 58, Triton X-100, CHAPSTM, deoxycholate and dodecyl sulfate.

15 45. The method of Claim 1, wherein at least two biopolymers are assayed simultaneously in the same sample.

46. The method of Claim 1, wherein said temperature is 15°C to 80°C.

47. The method of Claim 1, wherein said temperature is 40°C to 45°C.

20 48. The method of Claim 1, wherein said fixed specimen is contacted with said hybridization medium for about 5 minutes to about 240 minutes.

49. The method of Claim 1, wherein said method is accomplished within about 5 minutes.

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50. The method of any of Claim 1, wherein said probe comprises a mixture of short probes to multiple regions of the target polymer.

51. A method of detecting the presence of a biopolymer in a previously stained specimen having substantially intact cellular membranes by assaying cellular biopolymers comprising the steps of:

5 contacting said sample with a medium comprising a denaturing agent, a hybrid stabilizing agent, a buffering agent, a membrane pore-forming agent and at least one probe, said contacting being under hybridizing conditions in the presence of at least one detectable label; and

10 detecting hybrid formation by means of said label, without performing a prehybridization step for blocking nonspecific binding of said probe(s) and facilitating probe entry before contacting said sample with said medium.

52. The method of Claim 51, wherein said medium further comprises a fixative agent.

15 53. The method of Claim 51, wherein said specimen comprises a microorganism.

54. The method of Claim 51, wherein said hybrid formation is selected from the group consisting of duplex and triplex.

20 55. The method of Claim 51, wherein said probe has a nucleotide sequence at least substantially complementary to a specific target nucleotide sequence to be detected.

56. The method of Claim 55, wherein said target nucleotide sequence is about 75 bases.

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57. The method of Claim 53, wherein said microorganism is selected from the group consisting of bacteria, viruses and fungi.

58. The method of Claim 1, wherein said specimen comprises a eukaryotic cell.

5 59. The method of Claim 7, wherein said eukaryotic cell is a human cell.

60. The method of Claim 1, wherein said specimen comprises a cellular gene.

61. The method of Claim 60, wherein said cellular gene is selected from the group consisting of an oncogene, a tumor suppressor gene and a stimulating growth factor.

10 62. The method of Claim 61, wherein said oncogene is selected from the group consisting of c-erb-B-2, c-myc, c-myb and c-ras.

63. The method of Claim 61, wherein said tumor suppressor gene is selected from the group consisting of P-53 and retinoblastoma gene.

15 64. The method of Claim 61, wherein said stimulating growth factor is selected from the group consisting of transforming growth factor- α , epidermal growth factor and colony stimulating factor-granulocyte-macrophage.

20 65. The method of Claim 57, wherein said bacteria in said previously stained specimen is selected from the group consisting of *Streptococcus*, *Staphylococcus*, *Clostridium*, *Bacillus*, *Pseudomonas*, *Salmonella*, *Klebsiella*, *Bacteroides*, *Escherichia* *coil*, *Neisseria gonorrhea* and *Chlamydia*.

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66. The method of Claim 57, wherein said fungi are selected from the group consisting of *Candida*, *Cryptococcus neoformans* and *Blastomyces dermatitides*, *Histoplasma capsulatum*, *Coccidioides immitis* and *Paracoccidioides brasiliensis*.

5 67. The method of Claim 51, wherein said previously stained specimen is selected from the group consisting of a cell sample and a tissue sample.

68. The method of Claim 67, wherein said cell sample is selected from the group consisting of cervical cells, bone marrow cells, hepatocytes, cerebrospinal fluid cells, blood cells, oral mucosa cells, lung cells and skin cells.

10 69. The method of Claim 67, wherein said tissue sample is selected from the group consisting of lymph node tissue, mammary tissue, cervical tissue, colon tissue, prostate tissue, cardiac tissue and brain tissue.

70. The method of Claim 51, wherein said previously stained specimen was stained with a stain selected from the group consisting of a papanicolaou stain, a Wright stain, a Hematoxylin and Eosin stain and Diff-Quick stain.

15 71. The method of Claim 57, wherein said virus in said previously stained specimen is selected from the group consisting of human papilloma virus, herpes simplex virus II, hepatitis, human immunodeficiency virus, influenza virus, parainfluenza virus and rota virus.

72. The method of Claim 65, wherein said biopolymer is mRNA.

20 73. The method of Claim 65, wherein said biopolymer is DNA.

74. The method of Claim 65, wherein said biopolymer is 16S DNA.

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75. The method of Claim 65, wherein said biopolymer is selected from the group consisting of mRNA, a synthetic biopolymer, DNA and 16S rDNA.

76. The method of Claim 65, wherein said biopolymer is a synthetic biopolymer.

5 77. The method of Claim 71, wherein said biopolymer is mRNA.

78. The method of Claim 71, wherein said biopolymer is DNA.

79. The method of Claim 71, wherein said biopolymer is 16S DNA.

80. The method of Claim 71, wherein said biopolymer is a synthetic biopolymer.

10 81. The method of Claim 71, wherein said biopolymer is selected from the group consisting from mRNA, DNA, 16S rDNA and a synthetic biopolymer

82. The method of Claim 65, wherein said specimens are cervical cells.

83. The method of Claim 71, wherein said specimens are cervical cells.

84. The method of Claim 65, wherein said staining is a papanicolaou smear
15 stain.

85. The method of Claim 71, wherein said stain is a papanicolaou stain.

86. The method of Claim 51, wherein said label is attached to said probe.

87. The method of Claim 51, wherein said label is added after the hybrid formation is complete.

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88. The method of Claim 51, wherein said label is selected from the group consisting of radioactive labels, fluorescences, chemiluminescences and enzyme labels.

89. The method of Claim 51, wherein said label is a conjugate of avidin and streptavidin.

5 90. The method of Claim 52, wherein said fixative is selected from the group consisting of ethanol, methanol, acetone and formaldehyde.

91. The method of Claim 51, wherein said denaturing agent is selected from the group consisting of formamide, urea, sodium iodide, thiocyanate, guanidine, perchlorate, trichloroacetate and tetramethylamine.

10 92. The method of Claim 51, wherein said hybrid stabilizing agent is selected from the group consisting of sodium chloride, lithium chloride, magnesium chloride and ferric sulfate.

15 93. The method of Claim 51, wherein said pore forming agent is selected from the group consisting of Brij 35, Tween, Brij 58, Triton X-100, CHAPSTM, deoxycholate and dodecyl sulfate.

94. The method of Claim 51, wherein at least two biopolymers are assayed simultaneously in the same sample.

95. The method of Claim 51, wherein said method is capable of detecting as few as one copy of target biopolymer per cell.

20 96. The method of Claim 51, wherein said hybridizing conditions comprise a temperature of 15°C to 80°C.

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97. The method of Claim 51, wherein said hybridizing conditions comprise a temperature of 40°C to 45°C.

98. The method of Claim 51, wherein said method is accomplished within about 5 minutes.

5 99. The method of any of Claim 51, wherein said probe comprises a mixture of short probes to multiple regions of the target polymer.

100. A kit for detecting the presence of a biopolymer in a previously stained specimen having substantially intact cellular membranes by assaying cellular target biopolymers comprising,
10 a hybridization solution comprising a denaturing agent, a hybrid stabilizing agent, a buffering agent, and a membrane pore-forming agent.

101. The kit of Claim 100 further comprising,
 a supply of a probe, said probe capable of hybridizing with said target biopolymer to form a hybridized complex.

15 102. The kit of Claim 100 further comprising,
 means for contacting said suspect specimen with said probe to form said hybridized complex, and
 means for measuring for the presence of said probe.

20 103. The kit of Claim 100 further comprising,
 a detectable label capable of detecting hybrid formation.

104. The kit of Claim 103, wherein said detecting of hybrid formation is quantitative.

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105. The kit of Claim 103, wherein said detectable label is an energy emitting label.

106. The method of Claim 51, in which the contacting is carried out in solution.

5 107. The method of Claim 51, in which the detecting is carried out by flow cytometry.

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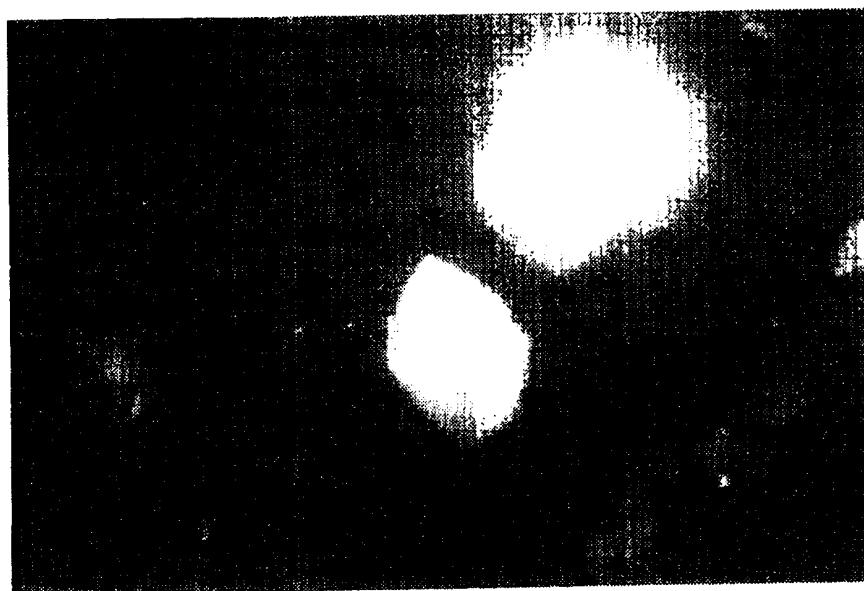


FIG. 1

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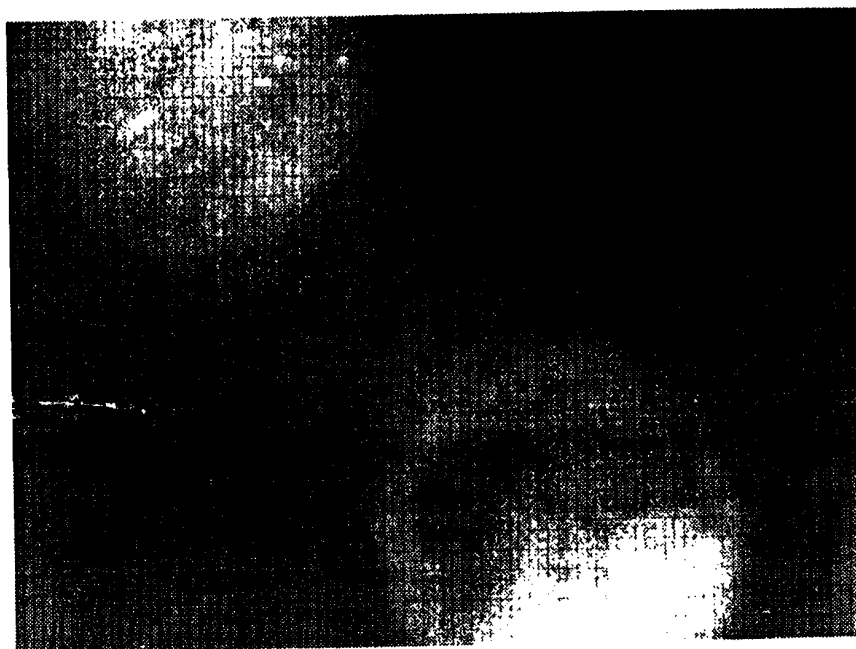


FIG. 2A

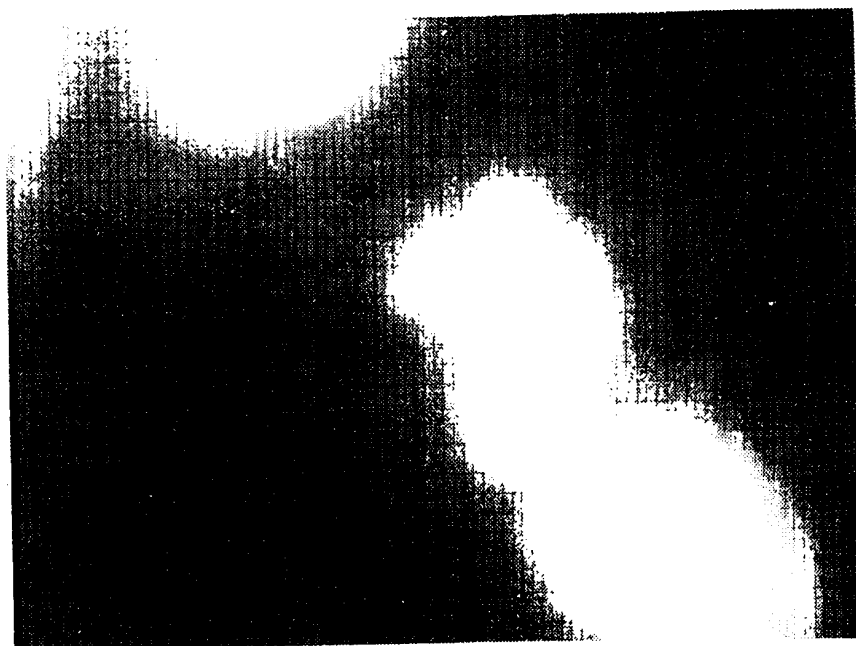


FIG. 2B

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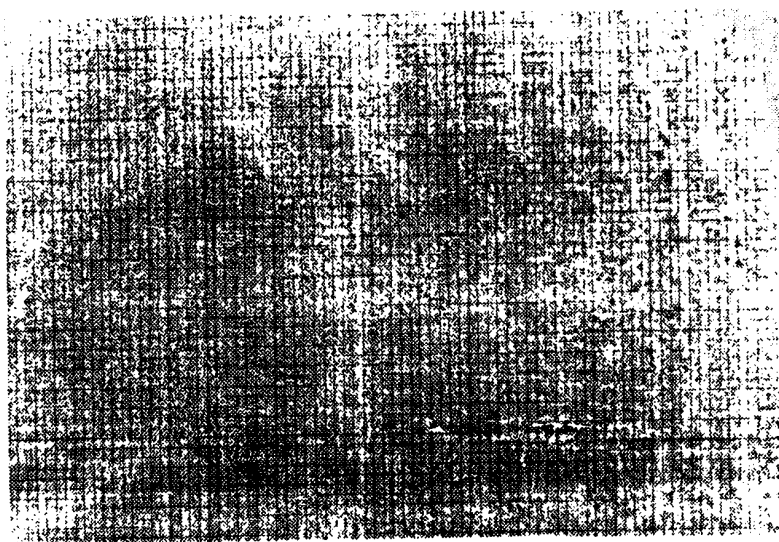


FIG. 3A

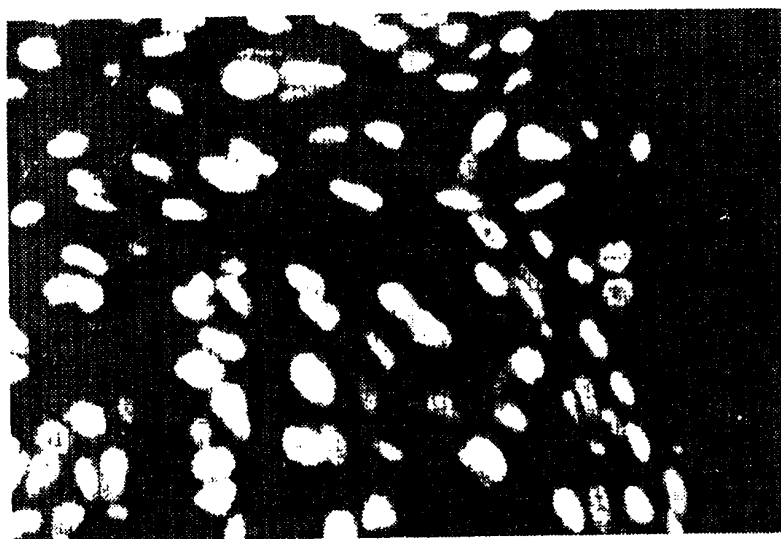


FIG. 3B



FIG. 3C

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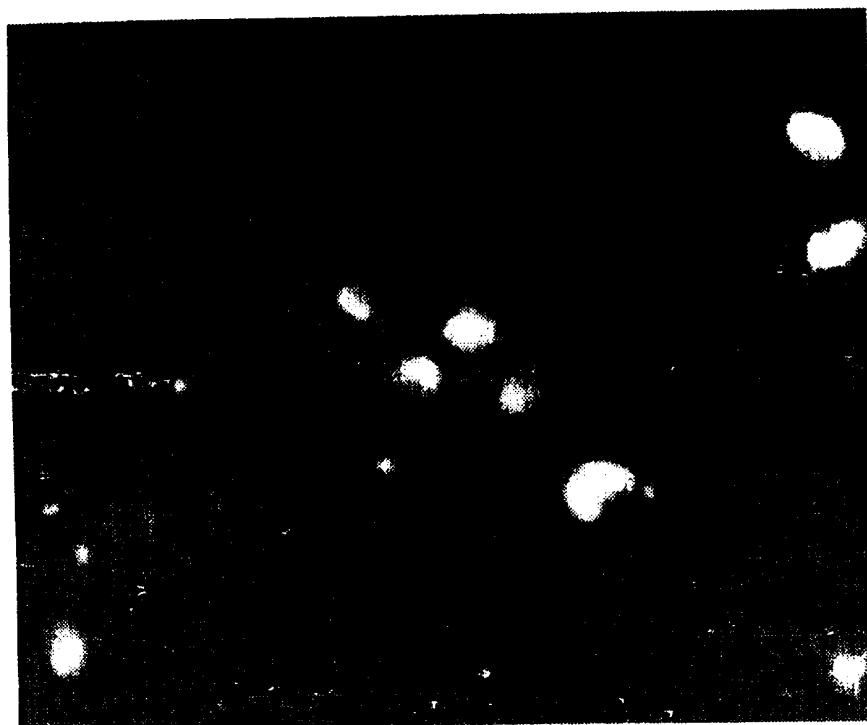


FIG. 4A

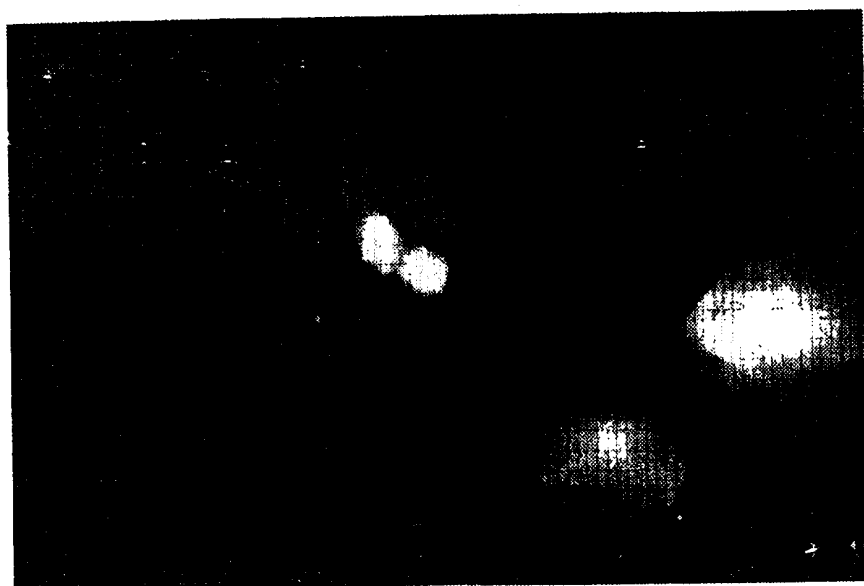


FIG. 4B

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FIG. 4C



FIG. 4D

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INTERNATIONAL SEARCH REPORT

International application No.

PCT/US93/06732

A. CLASSIFICATION OF SUBJECT MATTER

IPC(5) : C12Q 1/68; C07H 21/04

US CL : 435/6; 536/23.1, 24.31

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/6; 536/23.1, 24.31

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, MEDLINE, BIOSIS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X Y	THE JOURNAL OF HISTOCHEMISTRY AND CYTOCHEMISTRY, Volume 39, No. 6, issued 1991, X. Liang et al., "In situ hybridization with human papillomavirus using biotinylated DNA probes on archival cervical smears", pages 771-775, see entire document.	1-13,17-21,28,29,31, <u>33,35-47</u> 14-16, 22-27,30,48,49,51-100
X Y	US, A, 4,888,278 (Singer et al.) 19 December 1989, see entire document.	<u>101-106</u> 22,24,27,30,32,34,48-100,107

☒ Further documents are listed in the continuation of Box C.
 ☐ See patent family annex.

* Special categories of cited documents:	*T	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
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*E		earlier document published on or after the international filing date
*L		document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
*O		document referring to an oral disclosure, use, exhibition or other means
*P		document published prior to the international filing date but later than the priority date claimed
	*X	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
	*Y	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
	*G	document member of the same patent family

Date of the actual completion of the international search

23 AUGUST 1993

Date of mailing of the international search report

SEP 13 1993

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US93/06732

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	W.K. Joklik et al., eds., ZINSSER MICROBIOLOGY, published 1984, Appleton-Century-Crofts, (Norwalk, Connecticut), pages 444,445, 498, 499, 788, 789, 1134, 1135, 1150, 1151, 1184, see entire document.	14-16,22, 24,27,30,48,49